

A bacterial symbiont is converted from an inedible producer of beneficial molecules into food by a single mutation in the *gacA* gene

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Stable multipartite mutualistic associations require that all partners benefit. We show that a single mutational step is sufficient to turn a symbiotic bacterium from an inedible but host-beneficial secondary metabolite producer into a host food source. The bacteria's host is a "farmer" clone of the social amoeba *Dictyostelium discoideum* that carries and disperses bacteria during its spore stage. Associated with the farmer are two strains of *Pseudomonas fluorescens*, only one of which serves as a food source. The other strain produces diffusible small molecules: pyrrolnitrin, a known antifungal agent, and a chromene that potently enhances the farmer's spore production and depresses a nonfarmer's spore production. Genome sequence and phylogenetic analyses identify a derived point mutation in the food strain that generates a premature stop codon in a global activator (*gacA*), encoding the response regulator of a two-component regulatory system. Generation of a knockout mutant of this regulatory gene in the nonfood bacterial strain altered its secondary metabolite profile to match that of the food strain, and also, independently, converted it into a food source. These results suggest that a single mutation in an inedible ancestral strain that served a protective role converted it to a "domesticated" food source.

symbiosis | GacA–GacS two-component system | differential metabolomics

Small molecules regulate mutually beneficial associations between bacterial symbionts and their eukaryotic hosts. A fascinating set of structurally diverse molecules that defend the host, initiate host developmental changes, and carry out other important functions have been shaped by their evolutionary history (1–10). Recently, Brock et al. (11) described an association between the social amoeba *Dictyostelium discoideum* and a variety of Gram-negative bacteria, some of which it carries to initiate new food populations.

D. discoideum is a popular model for studying multicellularity, chemical signaling, general eukaryotic cellular mechanisms, and social phenomena (12–15). The protist is typically found in soil, where it preys on bacteria; in nutrient-rich environments it lives as single-celled organisms that reproduce by binary fission. Upon starvation, cAMP-mediated aggregation occurs, leading to the formation of a multicellular pseudoplasmodium containing up to 10⁵ individual cells. Eventually, the aggregate develops into a fruiting body in which some 20% of the cells differentiate into a dead stalk that supports a spherical structure known as the sorus; the latter contains 80% of the cells that turn into spores.

Previous work by Brock et al. (11) showed that about one-third of wild-collected clones of *D. discoideum* engage in stable associations with bacteria throughout the sporulation and dispersal process. These clones are called "primitive farmers" because they carry, seed, and prudently harvest their bacterial food.

Schultz and Brady (16) characterized agriculture as a specialized form of symbiosis known in only four animal groups: humans, bark beetles, termites, and ants. The fungus-farming ants, for example, collect material above ground and carry it to their underground fungal gardens. The ants and the fungi are

obligate mutualists; the fungus requires the ants, and the ants require the fungus (8). In addition, the ants have specialized anatomical features that contain bacteria (actinomycetes belonging to the genus *Pseudonocardia*) that produce small-molecule chemical defenses that protect the fungal crop from specialized fungal pathogens (5). This multipartite symbiosis has existed for ~50 million years and evolved into more than 230 species. The bark beetle system also involves an obligate mutualism between a fungal food source and a beetle host, along with *Streptomyces* spp. bacteria that provide chemical defenses, and this system has ~200 genera and ~6,000 species.

The initial model of primitive farming in *D. discoideum* contained a puzzling feature: only about half of the bacteria carried by the farmers served as food sources. Though it made sense that the farmer clones would carry bacterial food sources, it was not clear why they should also carry bacteria that they cannot eat. It seemed likely that we could resolve this question by exploring the differences between bacterial strains with similar genetic backgrounds but different functional roles.

In our study, we focused on a single tripartite association between one farmer *D. discoideum* clone and its two carried strains of the Gram-negative gammaproteobacterium *Pseudomonas fluorescens*. One of the *P. fluorescens* strains served as a food source for the farmer and the other one did not. In this study, we identified the role of the nonfood source in the symbiosis, as well as the genetic difference between food and nonfood *P. fluorescens*. Finally, a phylogenetic analysis allowed us to retrace the evolutionary history of the two bacterial strains.

Results and Discussion

Metabolomic and Structural Investigation of Symbiotic *P. fluorescens* Strains. To determine why *D. discoideum* would carry bacteria that did not serve as a food source, we investigated the production of secondary metabolites by the two symbiotic *P. fluorescens* strains associated with the host-farmer *D. discoideum* QS161 (from now on referred to as the "farmer"). Both symbionts had identical 16S rRNA gene sequences; however, one strain, *P. fluorescens* PfB-QS161 (from now on referred to as "PfB"), served as a food source, and the other one, *P. fluorescens* PfA-QS161 (from now referred to as "PfA"), did not (*SI Appendix*, Fig. S1). We used a differential metabolomics approach

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to identify molecules with potential functional roles in the symbiosis (Fig. 1).

We grew the food source strain PfB and the nonfood source strain PfA in liquid media for 24 h. Initial LC-MS analysis of the ethyl acetate extract of the PfA culture revealed two major peaks that correspond to two different compounds: a previously unreported compound and the known antifungal pyrrolnitrin (Fig. 1) (17). The extracts of the PfB culture revealed one major diffusible small molecule—namely, the iron chelator pyochelin (Fig. 1) (18). The latter contains three stereogenic centers of which one (C2'') can easily epimerize to yield a mixture of diastereoisomers known as pyochelin I and II. Furthermore, pseudomonads are known to produce two enantiomeric (mirror image) forms of pyochelin: (4'*S*, 4''*S*) and (4'*R*, 4''*R*), depending on whether they contain a cysteine epimerase domain in their biosynthetic gene cluster (19). Whereas fluorescent pseudomonads biosynthesize (4'*S*, 4''*S*) pyochelin, *P. aeruginosa*, biosynthesizes (4'*R*, 4''*R*) pyochelin (19). A measurement of the optical rotation revealed that PfB produces the enantiomer characteristic of fluorescent pseudomonads.

The unknown compound found in the PfA extract was produced in liquid culture at a concentration of $\sim 0.3 \mu\text{g}/\text{mL}$ ($1.3 \mu\text{M}$) after 24 h of cultivation at 30°C ; it was isolated as a pale yellow oil with the empirical formula $\text{C}_{14}\text{H}_{18}\text{O}_3$ as determined by high-resolution electrospray ionization-TOF mass spectrometry combined with both ^1H and ^{13}C NMR spectroscopy (SI Appendix, Table S1 and Figs. S2 and S3; Fig. 1). The ^1H NMR spectrum in deuterated methanol displayed three downfield protons at 6.33, 5.86, and 5.78 ppm, a double doublet at 4.51 ppm, and 12 aliphatic protons between 0 and 3 ppm. The ^{13}C NMR and gradient heteronuclear single-quantum coherence (gHSQC) spectra showed five aromatic, two olefinic, one oxygen-bearing aliphatic, and five aliphatic carbon signals. A combination of ^1H - ^1H correlation spectroscopy (COSY), gHSQC, and heteronuclear multiple-bond correlation (HMBC) spectra allowed us to identify

the molecule as the previously undescribed 3-ethyl-2-propyl-2*H*-chromene-5,7-diol (hereafter referred to as “chromene”; Fig. 1).

We confirmed the identity of both pyrrolnitrin and *enantiopyochelin* by a combination of high-resolution mass spectrometry and ^1H NMR spectroscopy data, which were compared with published data (SI Appendix, Fig. S5 and Table S2). We then investigated the role of the main secondary metabolites produced by the nonfood strain PfA in the symbiotic association with the farmer strain.

Differential Effect of Pyrrolnitrin on Farmer and Nonfarmer

***D. discoideum*.** In many symbiotic associations between bacteria and eukaryotes, the former produce secondary metabolites that protect their host from pathogens or display other beneficial effects. Pyrrolnitrin, which is produced by PfA, the nonfood source symbiont of *D. discoideum*, has potent antifungal activity against a variety of soil-borne fungi (20); it is also known to play a crucial role in other symbiotic associations between the producer *P. fluorescens* strain and plants. The decreased susceptibility of an infected host to soil-borne pathogens can be used as a form of biocontrol, and fluorescent pseudomonads play an important role as biocontrol agents (21–24). It is possible that Dictyostelia carrying pyrrolnitrin-producing strains like PfA could be similarly protected.

The discovery of robust pyrrolnitrin production by PfA ($0.4 \mu\text{g}/\text{mL} = 1.6 \mu\text{M}$ in a 24-h liquid culture) was in some ways also surprising because this metabolite is potentially toxic to many eukaryotes, including *D. discoideum*. In one recent study on predator-prey chemical warfare involving *P. fluorescens* as the prey and another amoeba (*Acanthamoeba castellanii*) as the predator, pyrrolnitrin was toxic, and supernatants of amoeba cultures strongly induced the production of the molecule (25). Farmer resistance and nonfarmer sensitivity would indicate that the farmers adapted to harboring a potentially lethal symbiont.

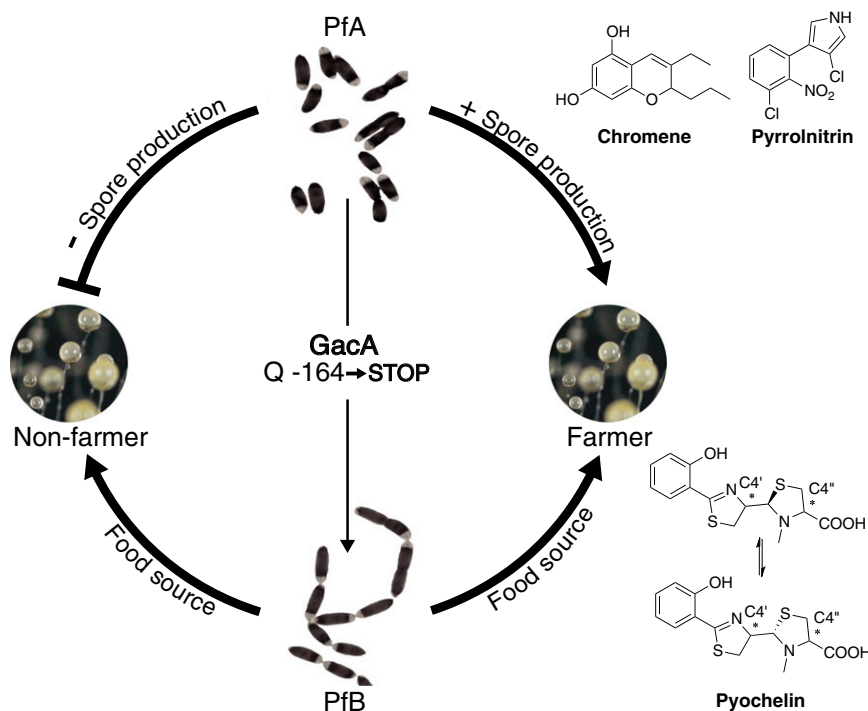


Fig. 1. Two strains are carried by the farmer *D. discoideum* QS161: *P. fluorescens* PfA (Upper) and PfB (Lower). Though the latter serves as a food source for the farmer, the former does not. Both strains produce different secondary metabolites: PfA produces a chromene and pyrrolnitrin, whereas PfB produces the putative siderophore (iron chelator) pyochelin.

To test whether the farmer has evolved the ability to resist the toxicity of its bacterial symbiont as a consequence of continuous exposure to its metabolites, we subjected the farmer *D. discoideum* strain to a range of concentrations of pyrrolnitrin. As a control, we investigated the effect of this compound on a nonhost, nonfarmer clone, *D. discoideum* QS160 (hereafter referred to as the “nonfarmer”), which was isolated in the vicinity of the farmer and was thus likely to have occasionally encountered the farmer *D. discoideum* strain as well as its symbiotic bacteria (PfA and PfB) and their secreted molecules. In *D. discoideum*, the number of spores produced from a given number of amoebas is a good indicator of the success of the social process, so we used this measure to evaluate the effect of pyrrolnitrin on both *D. discoideum* strains. To determine variation of spore production as a function of the concentration of pyrrolnitrin, we put vegetative amoebas in their exponential growth phase on a nitrocellulose filter paper soaked with starvation buffer containing different concentrations of pyrrolnitrin. Fruiting bodies form within 24 h; at this time point we counted the number of spores contained within the fruiting bodies.

The farmer strain and the nonfarmer strain performed differently in the presence of pyrrolnitrin (Fig. 2). Though the nonfarmer suffered from the toxic effect of pyrrolnitrin, the farmer did not, and in fact did better than controls. We interpret these results as an adaptation of the farmer to pyrrolnitrin’s toxic effects.

Differential Effect of Chromene on Farmer and Nonfarmer *D. discoideum*.

The presence of pyrrolnitrin in PfA suggested that chromene might have similar or related biocontrol activities. In a 24-h liquid culture, chromene was found to be present in comparable concentration to pyrrolnitrin (chromene: 0.3 $\mu\text{g/mL}$ = 1.3 μM ; pyrrolnitrin: 0.4 $\mu\text{g/mL}$ = 1.6 μM).

Initial bioassays, however, revealed no activity against the model fungus *Saccharomyces cerevisiae* or the gammaproteobacterium *Escherichia coli*, and only modest activity against *Bacillus subtilis* (IC_{50} = 82 $\mu\text{g/mL}$ = 35 μM). Because we found no obvious indirect effects (inhibition of *D. discoideum*’s microbial competitors) for chromene, we investigated possible direct effects on the farmer *D. discoideum*.

Treatment with chromene increased spore production in the farmer strain (Fig. 3), whereas the same treatment resulted in a decrease in spore production by the nonfarmer *D. discoideum* strain. The onset of visible differences in spore production occurred at concentrations on the order of 1 ng/mL (~ 4 nM). This concentration of chromene is likely to be ecologically relevant because it is ~ 300 -fold lower than the concentration of chromene in a 24-h culture of PfA.

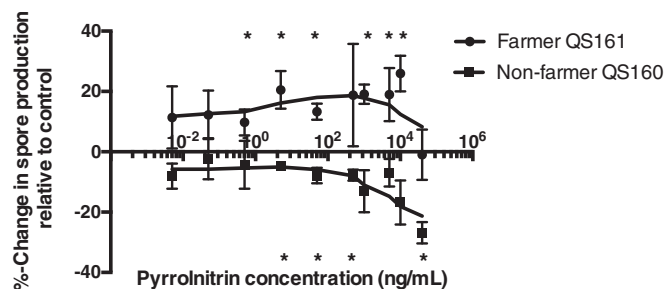


Fig. 2. Effect of pyrrolnitrin on farmer and nonfarmer *D. discoideum* QS161 and QS160, respectively. Each data point shows mean \pm SD at the specified concentration (three biological replicates; $P < 0.05$ two-tailed test paired t test of three experimental vs. three control values for each point, paired by experimental block, $df = 2$; *SI Appendix, Table S3*). For purposes of visualization, we fit the graph with a second-order smoothing polynomial with four neighbors on each side using GraphPad Prism 6 (www.graphpad.com) software.

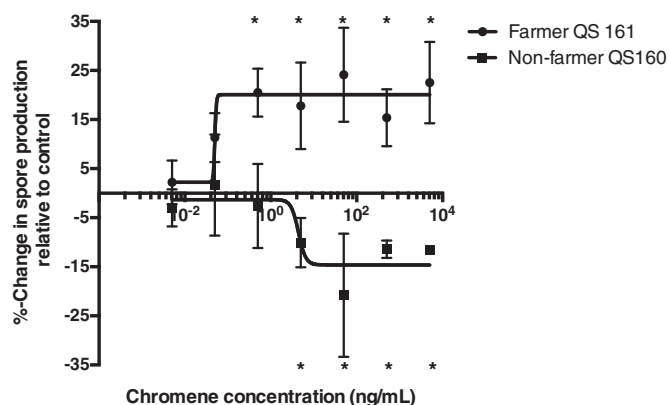


Fig. 3. Effect of chromene produced by PfA on the spore production of farmer *D. discoideum* QS161 vs. nonfarmer clone QS160. Each data point shows mean \pm SD at the specified concentration (four biological replicates; $P < 0.05$ two-tailed test paired t test of four experimental vs. four control values for each point, paired by experimental block, $df = 2$; *SI Appendix, Table S3*). For purposes of visualization, we fit the graph with a sigmoidal curve using GraphPad Prism 6 (www.graphpad.com) software.

Having determined the molecular basis for farmer-beneficial effects of the nonfood symbiont PfA, we investigated the genetic difference between this bacterium and the food source PfB.

A Single Mutation Determines both Chemical Profile Changes and Edibility, but They Are Distinct Pleiotropic Effects.

Because *Pseudomonas fluorescens* strains PfA and PfB both have identical 16S rRNA gene sequences, it seemed plausible that they shared a recent common ancestor. If so, only a few alterations in their genomes might have resulted in their observed phenotypic differences. Both PfA and PfB were thus characterized by Next Generation Illumina sequencing, and the resulting reads were assembled using the genome of Pf-5, which was sequenced in 2005, as a scaffold (26).

Because PfA and PfB had different secondary metabolite profiles, it seemed plausible to expect differences in biosynthetic genes of the two strains. We focused on the known biosynthetic gene clusters for the metabolites pyrrolnitrin and *enantio*-pyochelin. Though only PfA produces pyrrolnitrin, and only PfB produces *enantio*-pyochelin, both PfA and PfB had identical coding sequences for the biosynthetic gene cluster for pyrrolnitrin and *enantio*-pyochelin. This finding indicates that the difference between the small molecules produced by the two strains results from differences in regulation rather than biosynthetic potential. As a result, we searched for regulatory genes that, when mutated, could lead to the observed phenotypes. The search focused on two-component systems as the most likely regulators of gene expression in bacteria.

A canonical two-component system is composed of a histidine sensor kinase and a cognate response regulator. Upon stimulation, the sensor kinase undergoes autophosphorylation to yield a phosphorylated histidine, which in turn activates the response regulator by phosphotransfer to an aspartyl residue (27, 28). The response regulator then mediates the output, typically a change in gene expression (29).

We found a point mutation in PfB generating a premature stop codon that resulted in a truncated *gacA* gene product (Fig. 4). A cytosine-to-thymine mutation was responsible for the conversion of wild-type *gacA* glutamine-164 to a stop codon in PfB’s *gacA* gene.

The GacA–GacS system is a well-known global regulator in many pseudomonads. In the opportunistic pathogen *P. aeruginosa*, for instance, the GacS–GacA two-component system regulates the expression of acute and chronic virulence determinants (30). In

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PfA    MIRVLVDDHDLVRTGITRMLADIDGLQVVGQAESGEESLLKARELKPDDVMDVKMPGGIGGLEATRKLRRSHPD
PfB    MIRVLVDDHDLVRTGITRMLADIDGLQVVGQAESGEESLLKARELKPDDVMDVKMPGGIGGLEATRKLRRSHPD

PfA    IKVVAVTVCEEDPFPTRELLQAGAAGYLTGKAGLNEMVQAIRLVFAGQRYISPQIAQQLVFKSFQPSDDSPFDALSE
PfB    IKVVAVTVCEEDPFPTRELLQAGAAGYLTGKAGLNEMVQAIRLVFAGQRYISPQIAQQLVFKSFQPSDDSPFDALSE

PfA    REIQIAlMIVGCQKVQIISDKLCLSPKTVNTYRIRIFEKLSISSDVELTLLAVRHGMVDASL*
PfB    REIQIAlMIVGC*KVQIISDKLCLSPKTVNTYRIRIFEKLSISSDVELTLLAVRHGMVDASL*

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↑
HELIX TURN HELIX

STOP
DNA-binding motif

Fig. 4. Amino acid sequence alignment of the *gacA* gene product of both PfA and PfB shows that a premature stop codon in the *gacA* gene of PfB leads to a truncated protein. The latter is characterized by the loss of the highly conserved helix turn helix motif, which is required for DNA binding.

P. fluorescens, the GacA–GacS system is a global regulator for antibiotic production (31, 32). Upon binding to an as-yet-undefined external signal, this regulatory system upregulates the production of pyrrolnitrin, and downregulates the production of siderophores such as pyochelin. Spontaneous mutations disabling the GacA–GacS system have been reported in *P. fluorescens* that were mainly characterized by a change in colony morphology (33), not unlike the differences in morphology between the PfA and PfB strains in this study. Furthermore, such mutations in pseudomonads are known to occur in the rhizosphere of plants where they display a role in competitive root colonization (34). The point mutation observed in PfB introduces a premature stop codon upstream of the helix-turn-helix motif required for DNA binding, and inability to bind DNA prevents the GacS response regulator from activating transcription (35) (Fig. 4).

To verify that a mutation in this two-component system was sufficient to explain the chemical and edibility differences between PfA and PfB, we deleted the entire *gacA* gene in PfA. A clean deletion was obtained by allelic replacement (36). The *gacA* knockout of PfA displayed the PfB phenotype: it could now serve as a food source for the farmer *D. discoideum* (SI Appendix, Fig. S1) and the secondary metabolite profile was the same as for PfB (Fig. 5). However, a complementation of PfB with PfA supernatant (containing pyrrolnitrin and chromene) did not turn PfB into a nonfood source, so it seems unlikely that these two secreted molecules are responsible for the inedibility of PfA (SI Appendix, Fig. S10). Instead, the edibility difference between the strains must result from some other, unknown downstream effect of *gacA*, which is not surprising given that its inactivation affects expression of 10% of the genes in the genome (32).

A single mutation transforms a beneficial but inedible *P. fluorescens* strain into a food source for *D. discoideum*. The nonfood source produces secondary metabolites that provide chemical defenses (pyrrolnitrin) and increase spore production in the farmer (chromene and pyrrolnitrin). This mutation in the *gacA* gene generates independent but closely related symbiotic strains that perform strikingly different roles in the symbiosis.

Though the genetic difference between the two bacterial strains was determined, a detailed phylogenetic analysis is required to understand their evolutionary history.

The Evolution of Edibility. It seems likely that the functional *gacA* gene is ancestral because there are many more possible loss-of-function mutations than gain-of-function mutations. In support of this hypothesis, we examined the *gacA* sequences from the 11 other *P. fluorescens* strains with sequenced genomes and also the two additional *P. fluorescens* strains we have isolated from other *D. discoideum* farmer clones (PF-QS68, PF-QS152). All *gacA* sequences appeared functional; none had the stop codon at position 164 or any other evident disabling stop codon or frameshift-causing insertion or deletion.

Thus, the stop mutation that causes edibility is clearly derived. The similarity of PfA and PfB suggests they are very closely related, yet both of them display a significant number of SNPs (136 in 5,561 homologous genes) showing they diverged well before we isolated them in the laboratory. We then estimated the maximum-likelihood phylogeny using DNA sequences of 20 conserved genes for all 15 strains, using *Pseudomonas syringae* as an out-group. The result (Fig. 6) shows very clear support (100% bootstrap) for a clade that includes the previously sequenced Pf-5 along with the four strains collected from *D. discoideum*.

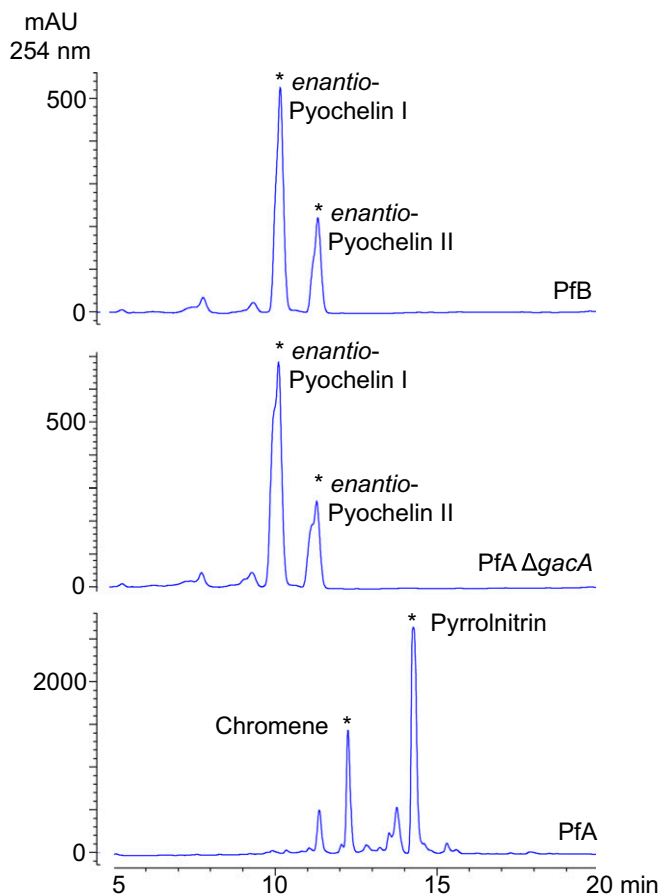


Fig. 5. HPLC (254 nm) trace of an ethyl acetate extract of PfB (Top), PfA $\Delta gacA$ (Middle), and PfA (Bottom) cultures. Though PfB and PfA $\Delta gacA$ display virtually identical traces, PfA is distinctly different: PfA produces chromene and pyrrolnitrin, whereas PfB and PfA $\Delta gacA$ both produce the enantiomers of pyochelin I and II.

There is some uncertainty (50% bootstrap) about the relative positions of the outermost clones in the clade (Pf-5 and Pf-QS68). This best tree is consistent with either two gains of the trait of being carried by *D. discoideum* or with a single gain plus a loss in Pf5, and because of uncertainty at the key node, we also cannot rule out a less-supported tree with Pf5 basal to the other four, with a single gain.

The tree also strongly supports PfA and PfB being the closest relatives whose sister group is another farmer-carried clone, Pf-QS152. Although the result could change with additional clone sampling, the most parsimonious conclusion is the edibility of PfB evolved in a strain already being carried by *D. discoideum*. This analysis suggests the unusual result that an organism evolved to be eaten by another, which makes sense only through kin-selected benefits to clonemates, more of whom will be carried to new locations by the well-fed farmer *D. discoideum* clone.

Conclusions and Perspective. The original description of primitive farming by the social amoeba *D. discoideum* distinguished two types of amoebas: farmers, which had fruiting bodies containing several types of bacteria, and nonfarmers, which were bacteria-free (11). Roughly half of the farmer-associated bacteria could serve as a food source, and farmers transported to bacteria-poor environments were able to sow food bacteria and eventually consume this bacterial food. Using a differential metabolomics analysis coupled with metabolite isolation and characterization, we showed that a nonfood source bacterial symbiont (PfA) produces secondary metabolites that are beneficial for the farmer. One of the metabolites, chromene, functions directly by potentially enhancing spore formation at ecologically relevant concentrations in the farmer strain, and suppressing spore formation in the nonfarmer strain. The other, pyrrolnitrin, showed similar effects, and it is likely to counter microbial pathogens of other species. The farmer strain was resistant to the inhibitory effects of pyrrolnitrin and chromene, which indicated that the farmer had adapted to carrying the nonfood strain PfA. Chemically uncharacterized supernatants collected from different non-food bacteria carried by other *D. discoideum* clones also help their

farmer clone and harm non-carrier clones (37). Taken together, these observations provide a strong argument for coevolution of both the farmer and symbionts; they also establish that the symbiosis is not just a food and farmer symbiosis, but rather displays some of the multipartite qualities of other farming symbioses, such as the fungus-farming ants and bark beetles with their crop fungi providing the food and the bacterial symbionts providing chemical defenses against fungal pathogens (5, 7–9). What distinguishes this farming symbiosis is the close relatedness of PfB and PfA—the bacterial food source and the small-molecule producer (although this close relatedness does not hold for bacterial symbionts of other *Dictyostelium* farmers). A single point mutation in the *gacA* gene is sufficient to completely alter the chemical repertoire of the nonfood source, thus effectively changing its role in the symbiotic association from secondary metabolite producer to food source.

Finally, these findings highlight the usefulness of investigating the small-molecule chemistry that underlies so much of bacterial–eukaryotic symbiotic associations as a source of both new chemistry (molecules and pathways) and biology (defense, development, cooperation, and evolution).

Materials and Methods

***P. fluorescens* and *D. discoideum* Growth.** We grew both *P. fluorescens* strains at 30 °C in SM/5 liquid media containing 2 g D-glucose, 2 g Bacto Peptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g MgCl₂, 1.9 g KH₂PO₄, and 1 g K₂HPO₄ per liter. We grew the farmer (QS161) and nonfarmer (QS160) *Dictyostelium discoideum* from spores on SM/5 agar plates (SM/5 liquid media supplemented with 15 g agar per liter) in association with *Klebsiella pneumoniae* as bacterial food source, at room temperature (21 °C) in Petri dishes (100 × 15 mm). For cocultures, we grew the farmer QS161 in association with its bacterial symbionts PfA and PfB (in a 1:10 ratio). For the complementation assay, we grew the farmer QS161 in association with PfB and 200 μL of cell-free PfA supernatant.

LC-MS Analysis of Bacterial Secondary Metabolites. A 1-L culture of the respective bacterial strain in SM/5 media was grown for 24 h at 30 °C and 200 rpm. After 24 h, we extracted 15 mL of the culture with 25 mL ethyl acetate (EtOAc). We separated the organic phase, dried it over Na₂SO₄, and concentrated it in vacuo. The cocultures were soaked with EtOAc, and the organic phase was separated by filtration and concentrated in vacuo. The crude concentrates were redissolved in 15% (vol/vol) methanol in water and applied to a Waters C18 Sep-Pak column (0.5 g) and washed with 15% (vol/vol) acetonitrile in water to remove polar molecules. The column was eluted with 100% acetonitrile, and solvents were removed in vacuo to yield a brown concentrate.

The concentrates were then subjected to HPLC-MS analysis using an Agilent HPLC system equipped with a diode array detector, and a 6130 Series quadrupole mass spectrometer was used with a Phenomenex Luna C18 (5 μm, 100 Å 100 × 4.6 mm, flow rate = 0.7 mL/min) column. We used the following gradient for HPLC-MS analysis: 0–1 min, isocratic 30% acetonitrile in water + 0.1% formic acid; 1–21 min, linear gradient from 30% acetonitrile in water + 0.1% formic acid to 100% acetonitrile + 0.1% formic acid.

Spore Assay. We prepared log-growth amoeba by plating 2 × 10⁵ spores of each clone in association with *K. pneumoniae* on SM/5 agar plates. Amoeba log growth occurs ~32–36 h after plating spores. At this time, we collected amoebas and washed them free of bacteria using ice-cold aqueous KK2 buffer (2.2 g KH₂PO₄ and 0.7 g K₂HPO₄ per liter). We determined amoeba density with dilution using a hemacytometer and a light microscope. For the filter-pad assay, we prepared dilutions of either chromene or pyrrolnitrin in KK2 + 0.5% DMSO from stock concentrations prepared in 100% DMSO. We used 150 × 15 mm Petri plates lined with two layers of Whatman no. 3 (Schleicher & Schuell) soaked with either control buffer or the various test dilutions laid with a grid of equidistant 13-mm square AABP 04700 (Millipore) black filter squares. To test social-stage spore production, we spotted the filters individually with 1.25 × 10⁶ amoebas in starvation buffer, and we prepared duplicate samples for each clone for each experiment. We allowed the clones to hatch, grow, and develop under direct light to limit potential movement of slugs before final culmination to fruiting bodies. Fruiting body formation for all clones was complete after ~24 h. We allowed the spores to mature in the fruiting bodies for an additional 24–48 h before collection. To determine spore number, we placed each test filter with fruiting bodies in an Eppendorf tube containing 1.0 mL KK2 + 0.1% Nonidet P-40 alternative (Calbiochem). The tubes were vortexed briefly to disperse the spores, and

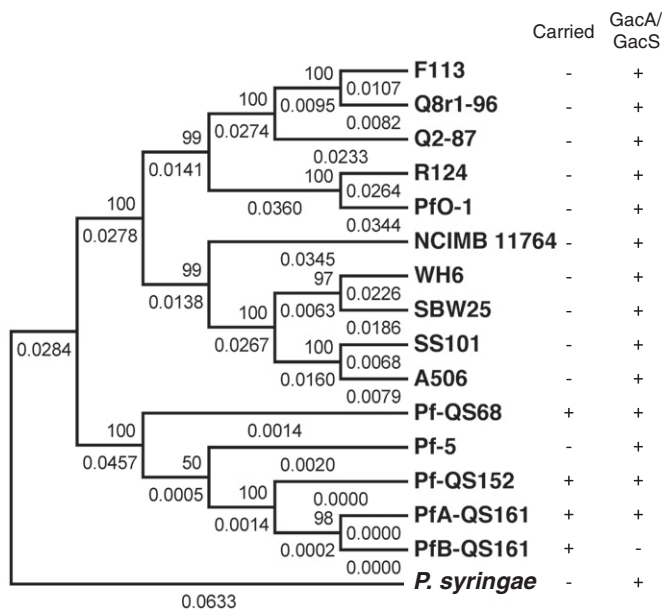


Fig. 6. Maximum-likelihood tree using 20 conserved genes. The numbers beneath branches show the branch length, and the numbers by the node show the bootstrap value of 500 replicates. Note that branch lengths are not drawn to scale and are very short in the bottom clade of five strains.

the spores were counted without dilution using a hemacytometer. We calculated spore number for experimental treatments as a percent change compared with control based on spore number recovered from starvation buffer control samples.

Sequencing. Genomic DNA of Pfa-QS161, Pfb-QS161, Pf-QS68, and Pf-QS152 was obtained from a 5-mL overnight culture at 30 °C in SM/5. Extraction of DNA was performed using GenElute Bacterial Genomic DNA Kit (NA2100; Sigma Aldrich). Next Generation Illumina sequencing with 100-bp single-end reads was performed at Harvard Medical School Biopolymers Facility. Reads were mapped to the reference genome sequence of Pf5 using Burrows-Wheeler Aligner software with default parameter settings (38). The SNPs and consensus genome sequences were called using SAMtools (39). A maximum-likelihood tree was constructed from concatenated DNA sequences of 20 genes (*dnaG*, *gcp*, *infB*, *ksgA*, *nusA*, *nusG*, *rplA*, *rplC*, *rplE*, *rplF*, *rplK*, *rplN*, *rpoB*, *rpsB*, *rpsC*, *rpsD*, *rpsG*, *rpsH*, *secY*, and *yehF*), a subset of the topologically congruent genes as reported by Bapteste et al. (40), from newly sequenced strains and other *P. fluorescens* strains with completed genome sequences that are retrieved from NCBI's BioProjects database (www.ncbi.nlm.nih.gov/bioproject/browse/) using the HKY model by MEGA5 (41). *P. syringae* pv. tomato strain DC3000 was included as an outgroup. We used DNA sequences rather than amino acid sequences because the group including the four *D. discoideum* carried clones, and Pf-5 differed by only one amino acid in these 20 genes.

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